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The long range goal of our research is to identify genes involved in the early stages of breast cancer development, specifically genes involved in the transition from hyperplasia to ductal carcinoma in-situ (DCIS) and DCIS to invasive breast cancer. The objective of this study was to investigate the role of LAF-4, a gene which we have recently identified as being transcriptionally perturbed in breast cancer, in the development of this disease. Analysis of LAF-4 expression in a group of breast tumors showed that in 24% of the ANN tumors, the level of LAF-4 mRNA was significantly higher than the highest level found in the normal tissues. In the remaining cases, the expression was barely detectable. We reviewed the histology of the cases and performed in situ hybridization analysis to confirm that the expression originated from the mammary carcinoma cells. We are in the process of determining by microdissection at which stage in progression expression becomes elevated. By studying these transitions it will be possible to identify key genes in the progression to malignancy and develop targeted approaches to breast cancer prevention.

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Introduction

Advances in prevention and treatment of breast cancer require an understanding of the etiology and pathogenesis of the disease. It has emerged that the evolution towards malignancy is driven by a progressive accumulation of genetic alterations. In effect, these alterations compromise the physiological activities of protooncogenes and tumor suppressor genes. Cumulatively, these genes account for a small proportion of cases of sporadic breast cancer so it follows that many important genes are yet to be identified. The long range goal of our research is to identify genes involved in the early stages of breast cancer development, specifically genes involved in the transition from hyperplasia to ductal carcinoma in-situ (DCIS) and DCIS to invasive breast cancer. By studying these transitions, it will be possible to identify key genes in the progression to malignancy and develop targeted approaches to breast cancer prevention. Our objective is to characterise the role of LAF-4 in the development of breast cancer. LAF-4 is a gene which we have recently identified as being transcriptionally perturbed in breast cancer and thus may play an important role in breast cancer development.

HYPOTHESIS/PURPOSE

The hypothesis of this work is that alterations in gene expression can compromise the physiological functions of the gene products in the cell, and thereby perturb the balance in growth and development, leading to tumorigenesis. In particular, the induction of LAF-4 may have an important role in the pathogenesis of breast cancer.

BODY

We have been characterizing the functions of this gene to clarify its role in breast cancer.

<u>Task 1</u> Tissue microdissection to collect a panel of samples of various types (hyperplasia, DCIS, invasive carcinoma, etc.) We identified and collected the cases for the study. Dr. Done, an anatomic pathologist performed histological evaluation of the cases. We began with invasive breast cancers, since

these were the largest specimen. These cases were evaluated for the selection of suitable areas for microdissection. They have been microdissected and RNA has been prepared.

a. Tissue microdissection

Molecular analysis of gene expression of solid tumors is largely based on mRNA analysis of crushed frozen tumor samples. Since most tumors are composed of a mixture of neoplastic cells together with inflammatory, stromal, endothelial and other cell types, molecular alterations acquired by neoplastic cells may be masked. Many methods of RNA extraction require large amounts of starting material which are not available from the lesions we will study. We have adapted a previously described method to allow it to be used for frozen tissue sections. Samples are microdissected from cyrostat sections placed on 2% agarose coated glass slides. Microdissected tissue is chosen to use cryostat rather than FFPE sections to minimize RNA degradation. Microdissected tissue is immersed in a freezing solution and rapidly freeze-thawed to lyse the cells. Aliquots can then be used directly in RT-PCR reactions without further purification. We have examined the effect of different tissue thickness and different tissue staining dyes. We estimate that a small microdissected region, containing no more than 200 cells can contain enough RNA for 80-100 RP-PCR reactions.

Task 2 Quantitative RT-PCR on microdissected samples

Given the limitation in material when working with tumors, we have used a quantitative RT-PCR approach towards evaluation of gene expression. For the evaluation of LAF-4 mRNA expression in breast tumors, tumor RNA is reverse transcribed into cDNA to serve as template in multiplex PCR reactions covering primers specific for LAF-4 as well as the house keeping gene β2m. The relative level of LAF-4 expression is indicated by the ratio of intensities of LAF-4 to the internal control β2m PCR products on polyacrylamide gel stained with ethidium bromide. All quantitation is performed by laser densitometry. For each tumor sample, PCR was performed at several different cycles of amplification to ensure that quantitation within the linear phase of PRC kinetics. We have designed primers and

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optimized the conditions for RT-PCR for LAF-4. We confirmed the appropriate reference gene for the control and selected cell lines to be used as controls. To evaluate whether expression of LAF-4 is altered in breast cancer, quantitative RT-PCR analysis of LAF-4 mRNA level was evaluated in ANN tumor tissue in comparison to corresponding normal mammary tissue. It was found to be up-regulated in 2/7 cases. Analysis of LAF-4 expression in a large cohort of ANN breast tumors showed that for the majority of tumors, the distribution of LAF-4 mRNA level was comparable to that found in normal mammary tissue. However, in 11/45 ANN tumors, the level of LAF-4 mRNA was significantly higher than the highest level found in the normal tissues. The work has been presented at several meetings (see reportable outcomes) and a manuscript is in preparation. The analysis of the small amount of material from microdissected specimens remains to be completed. These are tedchnically challenging experiments and we are working on methods for linear amplification of RNA to optimize the material fror these studies.

<u>Task 3</u>   RNA in-situ hybridization

RNA in-situ hybridization is a technique that allows localization of RNA within tissue sections and comparison of expression levels between adjacent histologically defined lesions. We optimized a technique for performing RNA in-situ hybridization on cryostat sections of rapidly frozen breast tissues. We collected a set of samples that were frozen as close as possible to the time of surgery to reduce the action of RNAses as much as possible. We constructed a riboprobe of approximately 1kb in length to allow a reasonable degree of specificity by ligating the fragments into a Bluescript vector and transcribing the sequence. Briefly 14μm frozen sections were placed on 'Superfrost Plus' glass slides. Large-scale minipreps were used to prepare DNA. Two batches of DNA were linearised, one for sense and one for antisense strands. The sample was passed through a Qiaex PCR spin column and resuspended in Tris 10mM. DNA was precipitated with 3M NaOAc and ethanol. After an ethanol wash the nucleic acids were resuspended in 10mM Tris. The RNA was transcribed and labeled at the same time. The sections were fixed in paraformaldehyde. A proteinase K digest allows access to the nucleic

acids within the cells and the slides are acetylated. Prehybridization was carried out at 60C. Hybridization follows at 50-60C with 10-25µl of probe in 5ml of hybridization solution. Washing with SSC, RNA and RNaseA was optimized to both the tissue and the probe. The probe was labeled with anti digoxigenin antibody. The detection system was NBT/BCIP which yields a purple precipitate when exposed to the alkaline phosphate attached to the digoxigenin antibody. Endogenous alkaline phosphatase was blocked first with levamisole. The slides were then washed and mounted. We have constructed riboprobes against LAF-4 and conducted several hybridisation experiments. We found that the gene was expressed in the mammary carcinoma cells, but the assay lacked sufficient specificity and we did not pursue this approach further.

<u>Task 4</u> Analysis of methylation status of promoter region

These experiments were scheduled for the third year of the grant. We planned to determine the mechanism underlying LAF-4 transcriptional activation in some breast tumors, with a specific focus on the status of DNA methylation. Since the LAF-4 sequence between the transcriptional start site and the first codon is GC rich, we hypothesize that the loss of DNA methylation may explain the presence of LAF-4 mRNA in some tumors. This would be tested using a combination of restriction enzymes that are methylation-sensitive, and PCR amplification.

KEY RESEARCH ACCOMPLISHMENTS

1. Selection of cases of pre-malignant breast lesions, in situ carcinomas and invasive breast cancers.

2. Development and refinement of methods for microdissection of cases of pre-malignant breast lesions, in situ carcinomas and invasive breast cancers. These techniques were optimized by Dr. Susan Done as a PhD student, using invasine cancer specimens with previously determined p53 status. The maunsucripts indicated below as number 1 and 2 in reportable outcomes resulted from that work.

3. Design of primers for RT-PCR and analysis of LAF-4 expression in invasive breast cancers.

4.   In situ hybridisation to determine that LAF-4 is expressed in mammary carcinoma cells.

REPORTABLE OUTCOMES

1.   Done, S., Arneson, N.C., Ozcelik, H., Redston, M., and Andrulis, I.L.   (2001) p53 Protein Accumulation in Non-invasive Lesions Surrounding p53 Mutation Positive Invasive Breast Cancer. Breast Cancer Research and Treatment 65: 111-118

2.   Done, S., Arneson, N.C., Ozcelik, H., Redston, M., and Andrulis, I.L. (2001) p53 missense mutations in high-grade DCIS not associated with invasive breast cancer. J Natl. Cancer Inst. 93

3.   Squire, J., Özçelik, H., and Andrulis, I.L. (2001) Investigating the genetics of cancer. *In:* Oxford Textbook of Oncology (2nd edition) Oxford University Press (in press).

Personnel - Dr. Susan Done obtained her PhD. Nona Arneson continues to work as a technician.

Abstracts and presentations, published or in press:

1.   Done SJ, To MD, Redston M and Andrulis IL.   LAF-4 a putative proto-oncogene ins overexpressed in human breast cancer. Proceedings of the American Association for Cancer Research (2000)41:442.

2.   LAF-4 a putative proto-oncogene is overexpressed in human breast cancer. Presented at the American Association for Cancer Research Annual Meeting, San Francisco, April 2000.

3.   Overexpression of LAF-4, a putative proto-oncogene, in breast cancer.   Presented at the Canadian Breast Cancer Research initiativeReasons for Hope Breast Cancer Research Conference, Toronto, 1999.

3.   To, M., Doyle, T., Gryfe, R., Gallinger, S., Redston, M., St. George-Hyslop, P., Andrulis, I. (2000)   Potential involvement of the Alzheimer's disease-associated presenilin-2 gene in tumorigenesis. Era of Hope, Department of Defence Breast Cancer Research Program Meeting. June 8-12, Atlanta, GA

4. Gokgoz, N., Woodgett, J., Sun, X., Bull, S., and Andrulis, I.L. (2000) Analysis of gene expression paterns in breast cancer by microarray technology. American Society of Human Genetics Annual Meeting, October 3-7, Philadelphia, PA

CONCLUSIONS

The objectives are to characterize the functions LAF-4 to determine how pertubations in its expression contribute to tumorigenesis. Our preliminary results clearly demonstrate potential involvement in breast cancer pathogenesis of a gene, LAF-4, isolated from the dd-PCR application. We are characterizing the functions of this gene to clarify its role in breast cancer. If this gene proves to have a significant role in breast cancer, it will be useful to evaluate its prognostic potential in breast cancer in long term studies. A clear understanding of the timing of transcriptional deregulation could allow the design of targeted prevention strategies and offers the potential of substantially reducing the number of new breast cancer cases in the future.